Imbalance of Antioxidant Enzymes in Tumor Cells and Inhibition of Proliferation and Malignant Features by Scavenging Hydrogen Peroxide

Lucía Policastro,[1] Beatriz Molinari,[1,2] Fernando Larcher,[3] Patricia Blanco,[4] Osvaldo L. Podhajcer,[2,4] Cristina S. Costa,[1] Paola Rojas,[1] and Hebe Durán[1,2]*

[1]*Radiobiology Department, National Atomic Energy Commission, Provincia de Buenos Aires, Argentina*
[2]*National Research Council, Buenos Aires, Argentina*
[3]*Department of Cell and Molecular Biology, CIEMAT, Madrid, Spain*
[4]*Leloir Institute, University of Buenos Aires, Buenos Aires, Argentina*

The aim of this study was to evaluate the endogenous alterations of the antioxidant enzymes in tumor cells and to specifically compensate the resulting changes in the levels of reactive oxygen species (ROS) to control the malignant growth. We determined and compared the activities of antioxidant enzymes and the levels of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in tumor cell lines with different degrees of malignancy, paired with regard to their origin (PB/CH72T4, PDV/PDVC57, and HBL-100/MCF-7). An increase in superoxide dismutase activity and a decrease in the activities of H_2O_2-detoxifying enzymes, as a function of malignancy, coupled with a rise in H_2O_2 and a decrease in $O_2^{\cdot-}$ were demonstrated. Treatment of cells with exogenous catalase showed a dose-dependent inhibition of proliferation. This inhibition was also demonstrated in several cell lines of different tissue origin and species, suggesting a general role of H_2O_2 in cell proliferation. Moreover, stable expression of human catalase in MCF-7 cells inhibited proliferation and also reverted malignant features. We conclude that H_2O_2 played a crucial and general role in the regulation of proliferation and that an endogenous imbalance in antioxidant enzymes could be a relevant event in the carcinogenesis process.

Key words: catalase; hydrogen peroxide; cell growth; malignancy

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$), are generated by all aerobic cells during normal oxygen metabolism. The concentration of these reactive species is tightly controlled by specific-scavenging systems, including antioxidant enzymes and low molecular weight antioxidants. The intracellular concentration of ROS results from their production and removal by antioxidant defenses.

Cumulative information has implicated ROS in the development of cancer [1,2]. Many chemical carcinogens have been shown to act through free radical metabolites [1]. Some tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), stimulate the endogenous production of free radicals in several cell types [3,4], whereas the tumor promoter benzoyl peroxide induces oxidative stress directly both in isolated mouse epidermal cells [3] and in mouse skin in vivo [5]. Free radical-scavengers protect against cancer development in animal models and may be chemoprotective in humans [1–3]. Conversely, chronic inflammatory states that involve oxidative stress are associated with the development of cancer [1,3]. However, the mechanisms by which ROS are involved in malignant transformation remain unclear.

Excessive production of ROS usually results in cytotoxic effects, but interactions of individual oxidant species with cell-control mechanisms that occur in the absence of cytotoxic effects are potentially involved in signaling processes [6]. In particular, ROS have been associated with signal transduction pathways related with cell proliferation and differentiation [7–14]. However, controversial results have been reported regarding the involve-

*Correspondence to: Comisión Nacional de Energía Atómica, Departamento de Radiobiología, Av. Gral. Paz 1499, (1650) San Martín, Provincia de Buenos Aires, Argentina.

Received 28 May 2003; Revised 14 October 2003; Accepted 5 November 2003

Abbreviations: ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SOD, superoxide dismutase; DMBA, 7,12-dimethyl-benz-(*a*)anthracene; FBS, fetal bovine serum; GPx, glutathione peroxidase; HPO, horseradish peroxidase; NBT, nitroblue tetrazolium; MTT, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide; senHCcDNA, sense cDNA human catalase; antHCcDNA, antisense cDNA human catalase.

DOI 10.1002/mc.20001

ment of either $O_2^{\cdot-}$ [8] or H_2O_2 [13,14] in cell proliferation. Regarding the physiological role of ROS, changes in the levels of antioxidant enzymes could induce a modification in the intracellular concentrations of each ROS, leading to alterations in the control mechanisms of cell proliferation. The data in the literature on the modulation of antioxidant enzymes in the carcinogenesis process are highly controversial. In several tumor tissues, decreased activities of antioxidant enzymes, including superoxide dismutase (SOD) and catalase, have been reported [15,16], but in other reports increased enzyme activities have been described [17,18]. Given the role of different ROS as signaling molecules, these controversies on the levels of antioxidant enzymes could be due to differences in the resulting levels of each ROS in each experimental model. In this sense, Gardner et al. [19] analyzed how SOD overexpression could affect the levels of H_2O_2 in a different way depending on the cellular context. Thus, to evaluate the involvement of antioxidant enzymes in the generation of the malignant phenotype, it would be relevant to consider the alterations in the balance between the different antioxidant enzymes and the levels of each ROS. In this sense, an imbalance in the antioxidant system in melanoma cells [20] and in lung cancer [21] has been reported. However, these reports [20,21] do not correlate the endogenous imbalance in the levels of antioxidant enzymes with variations in ROS concentrations and the control of tumor cell proliferation.

In the present study, three matched pairs of cell lines, which were compared with regard to their origin and their differences in malignancy, were used to evaluate the correlation between the activities of antioxidant enzymes and the levels of ROS. In these cell lines, we demonstrated an endogenous imbalance in antioxidant enzymes with a concomitant increase in H_2O_2 and a decrease in $O_2^{\cdot-}$ as a function of malignancy. Moreover, scavenging of H_2O_2 by exogenous addition of catalase inhibited proliferation. This inhibition was also demonstrated in several cell lines of different tissue origin and species, suggesting a general role of H_2O_2 in cell proliferation. Moreover, stable transfection of malignant cells (MCF-7) with cDNA of human catalase inhibited proliferation and also reverted malignant features.

MATERIALS AND METHODS

Cell Lines and Cell Cultures

All reagents and media for cell cultures were purchased from Invitrogen Argentina.

The cell lines used herein have different origins. Cell lines of equal origin and different degrees of malignancy were compared as follows.

PDV and PDVC57 originally derived from C57BL/6N mouse, PDV derived from newborn mouse epidermal keratinocytes treated in culture with the carcinogen 7,12-dimethyl-benz-(*a*)anthracene (DMBA), and PDVC57 were obtained by explanting a tumor induced by PDV in a syngeneic mouse [22,23]. PDVC57 cells are more tumorigenic and have more malignant phenotype features than PDV cells [22–24].

PB and CH72T4 were originally derived from SENCAR mice. PB was derived from culture of a papilloma obtained by two stage carcinogenesis (DMBA-TPA) [25]. CH72T4 was obtained by four passages in vivo of CH72 cells in nude mice (Fernando Benavides, personal communication), and CH72 cell line originally derived from a carcinoma obtained by two-stage carcinogenesis (DMBA-TPA). CH72T4 cells are highly tumorigenic and have lost the expression of epithelial markers. PB cells are morphologically indistiguishable from normal keratinocytes, respond to differentiation stimuli, and are very weakly tumorigenic [25].

Primary cultures of epidermal cells from newborn C57BL/6N and SENCAR mice were used as normal cells.

The human cell lines used were HBL-100 and MCF-7. HBL-100 cell line was developed from the milk of a healthy woman [26], presents markers of breast epithelium, and has been used widely as a near-normal model for breast epithelial cells [27]. The MCF-7 breast carcinoma cell line was established from the pleural effusion of a patient with breast adenocarcinoma [28].

Other cell lines of different origins were used: MCA3D (nontumorigenic mouse epidermal cells), Vero (monkey kidney epithelial cells), F98 (mouse glioblastoma cells) (a gift from Dr. R. Barth), UMR106 (rat osteosarcoma cells) (a gift from Dr. S.B. Etcheverry and Dr. A.M. Cortizo), and PANC-1 (human pancreatic carcinoma cells).

The PDV, PDVC57, CH72T4, and MCA3D cell lines were grown in Ham's F12 medium supplemented with aminoacids, 8% fetal bovine serum (FBS), and 50 μg/mL gentamicin. PB cells were grown in S-MEM with 8% Chelex 100 resin-treated FBS, aminoacids, and 50 μg/mL gentamicin. HBL-100, MCF-7, and PANC-1 were grown in RPMI 1640 supplemented with 8% FBS and 50 μg/mL gentamicin. UMR106 and F98 were grown in D-MEM with 8% FBS and 50 μg/mL gentamicin. All cells were grown at 37°C in a 5% CO_2 humidified atmosphere.

For primary cultures, epidermal cells were obtained from skin of newborn mice, and 2×10^6 cells from SENCAR mice and 5×10^6 cells from C57BL/6N mice were seeded in 35-mm plate dishes and grown in S-MEM with 8% Chelex 100 resin-treated FBS, aminoacids, and 50 μg/mL gentamicin.

The comparative pairs of cell lines previously described were characterized to validate the model of different degrees of malignancy. For this purpose, doubling time, clonogenicity, and capacity of

anchorage-independent growth in semi-solid medium [29] were evaluated.

Antioxidant Enzyme Assays

All reagents were purchased from Sigma-Aldrich Co.

To determine the antioxidant enzyme activities, cells grown in 100-mm dishes during 48 h were scraped and homogenized in 1 mL phosphate buffer 50 mM, pH 7.8. Cells were disrupted by freezing and thawing at −80°C and centrifuged at $20\,000 \times g$ 10 min at 4°C.

SOD, catalase, and glutathione peroxidase (GPx) were measured as previously described [30–32]. Briefly, total SOD was measured by the nitroblue tetrazolium (NBT) reduction assay with a xanthine/xanthine oxidase $O_2^{\cdot-}$ generating system. One unit of SOD activity was defined as the amount of enzyme that inhibits the rate of blue diformazan formation by 50%. Catalase activity was measured spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm. A unit of catalase is defined as the disappearance of 1 μmol H_2O_2/min ($E = 43.6\ M^{-1}\,cm^{-1}$). GPx was measured indirectly by spectrophotometrically monitoring the oxidation of NADPH at 340 nm in a coupled assay system containing glutathione and glutathione reductase, and with t-butyl hydroperoxide as the substrate. A unit of glutathione peroxidase is defined as the oxidation of 1 nmol NADPH/min ($E = 6.22\ mM^{-1}\,cm^{-1}$).

Protein concentration was measured by Lowry's method.

Each experiment was performed at least three times with triplicate measurements for each condition.

Production of H_2O_2 and $O_2^{\cdot-}$ by Tumor Cells

To evaluate the production of H_2O_2 and $O_2^{\cdot-}$, cells were seeded in complete medium in 35-mm cell culture dishes and grown for 24 h. The rate of H_2O_2 production was evaluated by measuring H_2O_2 released into the medium by the scopoletin/horseradish peroxidase (HPO) assay [33] adapted for tumor cells [34]. Briefly, cells were washed twice with PBS and incubated with 1 mL of complete medium without phenol red, and with 1 U of HPO and 35 μM scopoletin. The fluorescence intensity of the cell media was measured at different times of incubation up to 4 h in a spectrofluorometer with excitation wavelength at 360 nm and emission wavelength at 460 nm. Control values of spontaneous fluorescence decay were obtained by incubating cells in absence of HPO. Standard curves were obtained with known amounts of H_2O_2. The production of H_2O_2 was expressed as nmol/h/5×10^5 cells. The rate of $O_2^{\cdot-}$ production was determined by measuring the reduction of NBT as previously described [35]. NBT reduction was determined spectrophotometrically at 640 nm and the production of $O_2^{\cdot-}$ was expressed as nmol of reduced NBT/h/5×10^5 cells. Cell number was determined by direct cell count at the same time of the measurements of H_2O_2 or $O_2^{\cdot-}$ production.

Each experiment was performed at least three times with triplicate measurements for each condition.

Treatments With Exogenous Catalase and Cell Growth Assays

Cells growing in 24-well plates or 35-mm dishes were treated with 0–1000 U catalase/mL and cell growth was evaluated after 24 h of incubation. Cell growth was determined in cells treated with catalase by direct cell count, by the 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) growth assay [36] and after 6 or 24 h by immunolabeling following BrdU incorporation [37]. For the MTT assay, the cells were incubated with 1 mL of cell culture medium with 200 μg/mL MTT for 2 h at 37°C. MTT medium was removed, and the formazan was solubilized with 1 mL DMSO and measured spectrophotometrically at 555 nm. Cell counts and the MTT assay were also performed in control cells 24 h after seeding to calculate the cell proliferation rate after 24 h of treatment. Results were expressed as percentage of inhibition of proliferation, referred to control cells without treatment.

For BrdU incorporation, cells were incubated with 20 μM BrdU for 2 h. Cells were subsequently fixed and treated with formamide 70% 2 h at 65°C. BrdU was detected by immunocytochemistry [37]. Results were expressed as percentage of positive cells.

Cells growing in 24-well plates were treated with 0–1000 U catalase/mL. After 24 h of treatment, cells were washed with PBS and incubated with fresh medium for 24 h. Cell number was evaluated by MTT assay as described above. Results were expressed as the increase in MTT reduction relative to control cells without treatment.

Apoptosis was evaluated by staining with Hoechst and by DNA laddering. Cells treated with 100 μM genistein were used as positive control.

Variations in the levels of H_2O_2 by treatments with 0–1000 U/mL catalase were evaluated by a slightly modified scopoletin/HPO assay. Cells were incubated with 0.5 U/mL HPO and 100 μM scopoletin and fluorescence intensity was measured after 24 h. Results were expressed as percentage of the H_2O_2 production of control cells.

All experiments were performed at least three times with duplicate or triplicate measurements for each condition.

Transfection of cDNA of Human Catalase in MCF-7 Cells

Plasmid pLK440 carrying the full-length cDNA of human catalase (kindly donated by Dr. Y.S. Ho, Institute of Chemical Toxicology, Wayne State

University, Detroit, MI) was digested with *Sal* I and the 1.8 kb human catalase cDNA insert was cloned into pBabe-neo retroviral vector [38]. The sense (senHCcDNA) and antisense (antHCcDNA) cDNA human catalase recombinant vectors were used to transfect MCF-7 cells by the lipofectamine method (Invitrogen Argentina) and stable clones were obtained by selection with 700 μg/mL geneticin. The catalase activity and the rate of H_2O_2 production of the isolated clones were evaluated by the assays described above. The proliferation capacity and the malignant features of these clones were characterized. Proliferation was evaluated by cell count and by immunolabeling of BrdU incorporation and malignancy was evaluated by the clonogenicity assay and the capacity of anchorage-independent growth [29].

Statistical Analysis

Data are presented as mean ± SD. Statistical analyses were performed with a one factor analysis of variance (ANOVA) and Tukey's method for multiple comparison. Regression analyses were performed for experiments of growth inhibition as a function of catalase dose.

RESULTS

Antioxidant Enzyme Activities

Table 1 shows the characterization of the comparative cell lines derived from SENCAR and C57BL/6N mice and from human mammary epithelium. The more malignant the cell line, the higher its clonogenic capacity and anchorage-independent growth (Table 1). These results are in agreement with previous descriptions of the cell lines used in this study [22–28] and confirm the predetermined degrees of malignancy of all the pairs of cell lines.

In order to evaluate the variations in antioxidant enzymes as a function of malignancy, we evaluated the activities of total SOD, catalase, and GPx in the different cell lines (Table 2). SOD activity was significantly higher in the more malignant cell lines, except for the human cell lines, while the activities of H_2O_2-detoxifying enzymes, catalase and GPx, decreased significantly as a function of malignancy. These data revealed an imbalance in antioxidant enzymes associated with malignancy as shown by the increase in the SOD/catalase and SOD/GPx ratio as a function of malignancy (Table 2).

Production of H_2O_2 and $O_2^{\cdot-}$

Due to the imbalance in the antioxidant enzymes, we expected an increase in the constitutive levels of H_2O_2 and a decrease in the constitutive levels of $O_2^{\cdot-}$. Thus, the production of H_2O_2 and $O_2^{\cdot-}$ in the different cell lines were measured (Figure 1). The results showed a significant increase in H_2O_2 production, that is, PB vs. CH72T4 ($P \leq 0.001$), PDV vs. PDVC57 ($P \leq 0.01$), and HBL-100 vs. MCF-7 ($P = 0.001$) and a significant decrease in $O_2^{\cdot-}$ production, that is, PB vs. CH72T4 ($P = 0.001$), PDV vs. PDVC57 ($P = 0.001$), and HBL-100 vs. MCF-7 ($P \leq 0.001$), as a function of malignancy.

Inhibition of Cell Proliferation by the Exogenous Modulation of H_2O_2 Levels

To establish the relationship between H_2O_2 levels and proliferation, the different cell lines were treated with different concentrations of catalase. Figure 2 shows the inhibition of proliferation induced by catalase in cell lines derived from SENCAR and C57BL/6N mice and in human cells. Regression analysis demonstrated a dose-dependent growth inhibition for all the cell lines, as evaluated by MTT assay (Figure 2A,C,E): PB ($R = 0.92$, $P \leq 0.001$), CH72T4 ($R = 0.95$, $P \leq 0.001$), PDV ($R = 0.96$, $P \leq 0.001$), PDVC57 ($R = 0.94$, $P \leq 0.001$), HBL-100 ($R = 0.99$, $P \leq 0.001$), MCF-7 ($R = 0.97$, $P = 0.004$) and by cell counts: PB ($R = 0.92$, $P \leq 0.001$), CH72T4 ($R = 0.91$, $P \leq 0.001$), PDV ($R = 0.97$, $P \leq 0.001$), PDVC57 ($R = 0.95$, $P \leq 0.001$), HBL-100 ($R = 0.97$, $P = 0.003$), MCF-7 ($R = 0.98$, $P \leq 0.001$). A significant inhibition of cell proliferation was demonstrated in all the cell lines after 6 and 24 h of catalase treatment as assessed by BrdU incorporation (Figure 2B,D,F), that is, PB 6 h ($P = 0.001$), 24 h ($P \leq 0.001$); CH72T4 6 h ($P = 0.037$), 24 h ($P = 0.002$); PDV 6 h ($P \leq 0.001$), 24 h ($P \leq 0.001$); PDVC57 6 h

Table 1. Characterization of the Comparative Cell Lines

Cell lines	Doubling time (h)	Clonogenicity (colonies/10^3 cultured cells)	Anchorage independence (colonies/10^4 cultured cells)
PB	34.3 ± 4.7	10 ± 2	0
CH72T4	18.7 ± 0.6*	350 ± 23†	52 ± 5†
PDV	18.9 ± 1.1	300 ± 35	2.3 ± 0.5
PDVC57	18.1 ± 2.1	401 ± 21*	22.5 ± 3†
HBL-100	30 ± 3.4	90 ± 17	9.2 ± 2
MCF-7	25 ± 2.4	180 ± 20†	65.6 ± 5†

Characterization of the comparative cell lines derived from SENCAR mice (PB and CH72T4), C57BL/6N mice (PDV and PDVC57), and from human mammary epithelium (HBL-100 and MCF-7).
*$P < 0.01$ compared with the other cell line of the same origin.
†$P < 0.001$ compared with the other cell line of the same origin.

Table 2. Activities of Antioxidant Enzymes

Cell lines	SOD (U/mg prot)	Catalase (U/mg prot)	GPx (U/mg prot)	SOD/catalase	SOD/GPx
Primary culture (SENCAR)	28 ± 1	18 ± 3.4	60.5 ± 2.5	1.55	0.46
PB	52 ± 2.4*	16 ± 1.9	49 ± 2.5	3.25	1.06
CH72T4	102 ± 27*,†	6.9 ± 1.3*,†	7.8 ± 0.4*,†	14.7	13
Primary culture (C57BL/6N)	61 ± 5.4	17 ± 9.8	46.5 ± 4.5	3.58	1.31
PDV	115 ± 9.5*	1.8 ± 0.5†	10 ± 0.1*	63	11.5
PDVC57	220 ± 26*,†	2.7 ± 0.2*	6.2 ± 1.3*	81	35.5
HBL-100	20 ± 5	5.4 ± 1.8	20.8 ± 2.9	3.7	0.96
MCF-7	41.5 ± 11	1.4 ± 0.48†	3.44 ± 0.7†	29.2	12

SOD, superoxide dismutase; GPx, glutathione peroxidase.
Activities of SOD, catalase, and GPx in cell lines derived from SENCAR and C57BL/6N mice and from human mammary epithelium, and in primary culture of epidermal cells from SENCAR and C57BL/6N mice.
*$P < 0.01$ compared with primary culture.
†$P < 0.01$ compared with the other cell line of the same origin.

($P \leq 0.001$), 24 h ($P \leq 0.001$); HBL-100 6 h ($P = 0.002$), 24 h ($P \leq 0.001$); and MCF-7 6 h ($P = 0.015$), 24 h ($P = 0.005$). The inhibition of proliferation was completely eliminated by prior heat inactivation of catalase.

The catalase dose–dependent inhibition of cell growth was confirmed in cells of different tissue and species origin (R = 0.88–0.95, $P \leq 0.001$) (Figure 2G), suggesting that the inhibition of proliferation by scavenging H_2O_2 is a general mechanism.

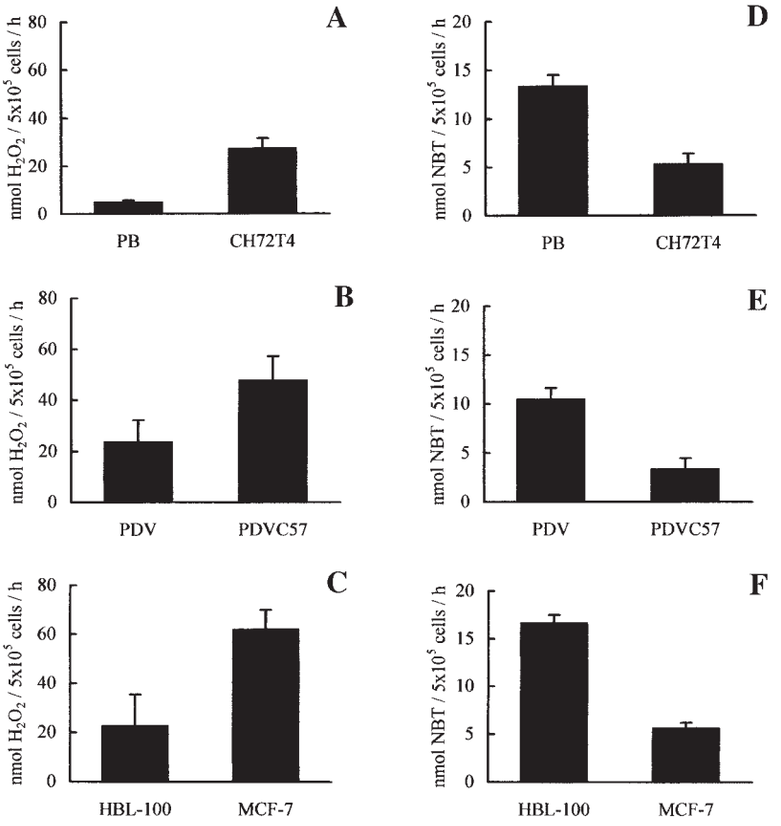

Figure 1. Production of H_2O_2 by tumor epidermal cells measured by the scopoletin/horseradish peroxidase assay and expressed as nmol $H_2O_2/5 \times 10^5$ cells/h (A, B, C). Production of $O_2^{\cdot-}$ determined by the NBT reduction assay and expressed as nmol of reduced NBT/5×10^5 cells/h (D–F). Cell lines derived from SENCAR mice (A, D), cell lines derived from C57BL/6N mice (B, E) and human mammary cell lines (C, F). Data are expressed as mean ± SD.

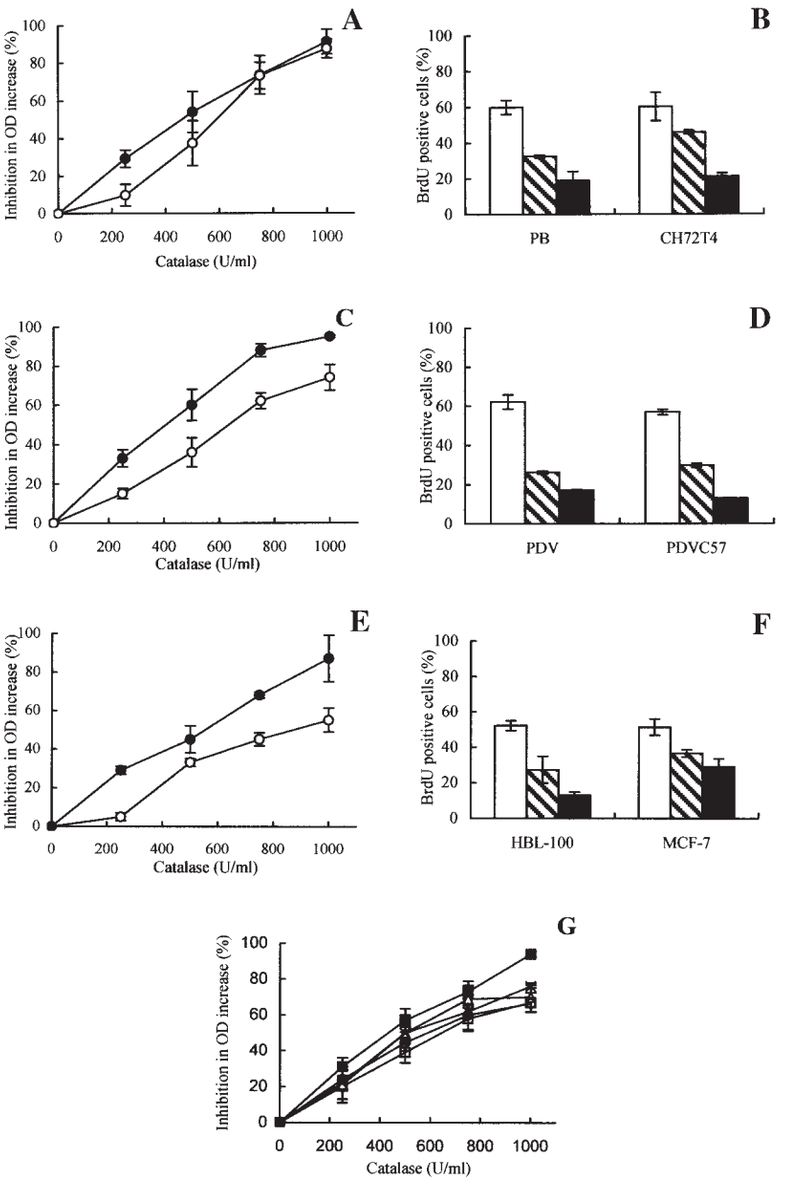

Figure 2. Inhibition of the increase in MTT reduction in control cells induced by exogenous catalase in cell lines derived from SENCAR mice (A, B), from C57BL/6N mice (C, D), from human mammary epithelium (E, F), and in other cell lines of different origin (G). (A, C, E, G) MTT growth assay after 24 h of catalase treatment, (●) PB, PDV, and HBL-100 cells, (○) CH72T4, PDVC57, and MCF-7 cells, MCA3D (■), Vero (×), PANC1 (○), F98 (□), and UMR106 (△). Similar results were obtained by direct cell count after 24 h of catalase treatment. Line drawings of the inhibition determined by direct cell count as a function of catalase dose were not included for the sake of brevity. Results of regression analysis for both methods were detailed in the text. (B, D, F) BrdU incorporation after 6 (▨) and 24 h (■) of catalase treatment (1000 U/mL), (□) control cells. Data are expressed as mean ± SD.

As expected, catalase treatments inhibited H_2O_2 production in a dose-dependent manner. Figure 3 shows the decrease in the production of H_2O_2 ($P \leq 0.001$) with the concomitant increase in the inhibition of proliferation in the human cell lines, HBL-100 and MCF-7.

Evaluation of Cytotoxicity and Apoptosis

Interestingly, the inhibition of cell proliferation observed upon catalase addition was reverted when treated cells were washed and incubated with fresh medium (Figure 4). In all cases, no significant differences in growth rate values were observed 24 h later. These results indicated that the inhibition was not due to a cytotoxic effect. Regarding the induction of apoptosis, there was no increase in the number of apoptotic cells in catalase (500 and 1000 U/mL) treated cultures, as compared with nontreated cultures. In all the cases, the number of apoptotic cells determined by staining with Hoechst

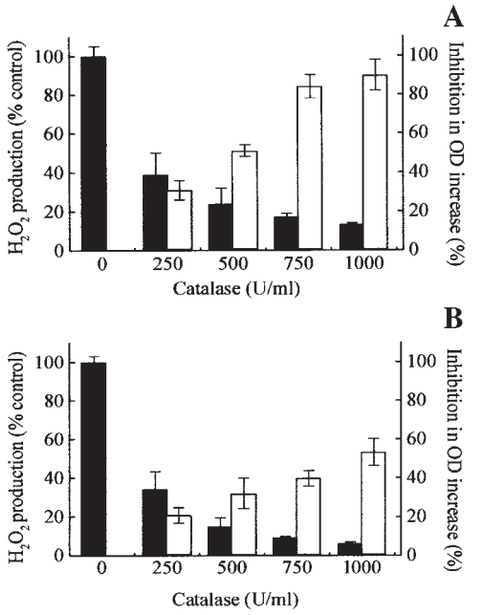

Figure 3. Hydrogen peroxide (H_2O_2) production (■) and inhibition of the increase in MTT reduction in control cells (□) as a function of catalase concentration after 24 h of treatment in human cell lines, (A) HBL-100 and (B) MCF-7. Data are expressed as mean ± SD.

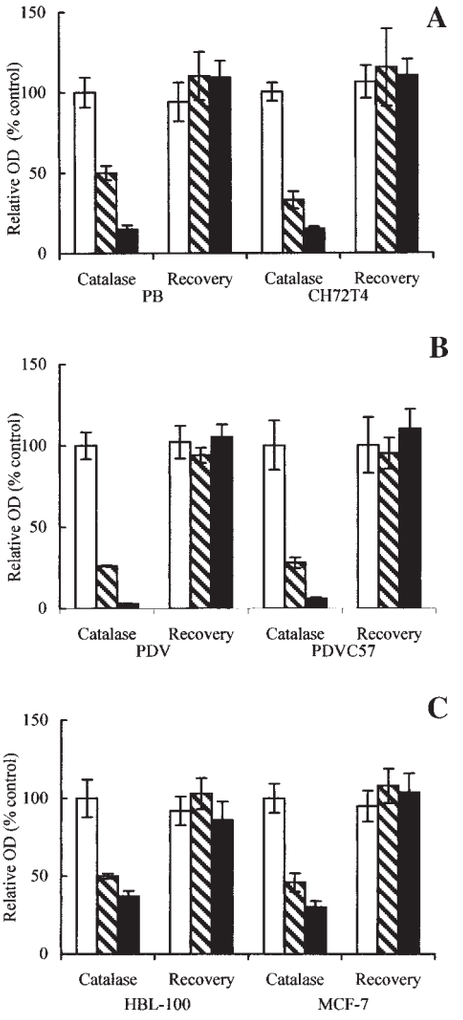

Figure 4. Recovery of cell proliferation after treatment with catalase. Cells treated with catalase for 24 h were washed and incubated for 24 h with fresh medium to evaluate recovery. Cell number was evaluated by the MTT assay. Results were expressed as the increase in MTT reduction relative to control cells without treatment. (A) PB and CH72T4 cells, (B) PDV and PDVC57 cells, and (C) HBL-100 and MCF-7 cells. (□) Control, catalase (▨) 500 U/mL, and (■) 1000 U/mL. Data are expressed as mean ± SD.

was less than 2%. Negative results for catalase-treated cells were obtained when apoptosis was determined by DNA laddering. Thus, the overall data strongly suggest that catalase is inducing a cytostatic effect.

Stable Expression of Catalase in Malignant Mammary Cells Following Gene Transfer

In order to confirm the previous data on the effect of catalase on cell proliferation, we stably transfected MCF-7 cells with the full-length human catalase cDNA (senHCcDNA). Parental MCF-7 cells and cells transfected with an antisense cDNA of human catalase were used as controls. Catalase activity of MCF-7 cells stably transfected with senHCcDNA was at least three times higher than for control cells (Figure 5A). Remarkably, a dramatic decrease in H_2O_2 levels was observed in MCF-7 cells stably expressing the catalase gene compared to control cells (Figure 5B).

Inhibition of Proliferation and Malignant Features by the Stable Transfection of cDNA of Human Catalase in MCF-7 Cells

Stable expression of catalase in MCF-7 cells led to a marked decrease in the proliferation capacity of MCF-7 cells as evaluated by their doubling time and BrdU incorporation (Table 3). Moreover, stable catalase expression also induced a dramatic decrease in MCF-7 cells clonogenicity and capacity of anchorage-independent growth, demonstrating a marked inhibition of their malignant phenotype (Table 3).

DISCUSSION

In the present study we demonstrated an endogenous imbalance of antioxidant enzymes coupled to a rise in H_2O_2 and a decrease in $O_2^{\cdot-}$ in tumor cell lines, which correlates with the degree of malignancy. Scavenging of H_2O_2 with exogenously added catalase inhibited cell proliferation both in near normal cells and in malignant cells. The involvement of H_2O_2 in proliferation was also demostrated in cell lines of different tissue origin (epithelial, fibroblast, glia, bone) from several species (mouse, rat, hamster, human), supporting the general and crucial role of H_2O_2 in the control of cell proliferation.

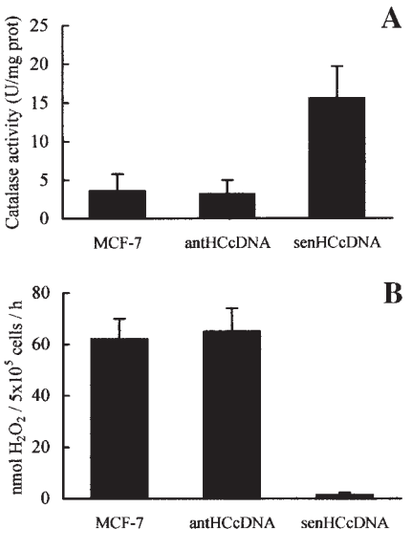

Figure 5. Characterization of stably transfected MCF-7 cells with the full-length human catalase cDNA (senHCcDNA), parental MCF-7 cells, and cells transfected with an antisense cDNA of human catalase (antHCcDNA). (A) Catalase activity determined spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm. (B) Production of H_2O_2 measured by the scopoletin/horseradish peroxidase assay and expressed as nmol $H_2O_2/5 \times 10^5$ cells/h. (C) Production of $O_2^{\cdot-}$ determined by the NBT reduction assay and expressed as nmol of reduced NBT/5×10^5 cells/h. Data are expressed as mean ± SD.

Moreover, stable expression of human catalase in MCF-7 cells inhibited cell proliferation and reverted malignant features.

McCord suggested that a cell producing a permanent oxidative shift in the redox status may undergo continuous proliferation that could be, in turn, a crucial event in the appearance of the malignant phenotype [39,40]. In this sense, Suh et al. [41] reported that fibroblasts overexpressing Nox1 (NADPH oxidase, which generates $O_2^{\cdot-}$ with secondary generation of H_2O_2) exhibit a transformed phenotype, including increased proliferation and aggressive tumor formation in athymic nude mice. To analyze if tumor cells have a constitutive oxidative shift, we evaluated the levels of antioxidant enzymes in epithelial cell lines with different degrees of malignancy. It is important to emphasize that in this study the alterations in antioxidant enzymes were evaluated as a function of malignancy in cells that have not been genetically manipulated to induce the malignant phenotype. These variations were compared with due regard for the origin of the cells. The results demonstrated an imbalance in the antioxidant system in the three comparative series of cell lines, with an increase in SOD and a decrease in H_2O_2-detoxifying enzymes, catalase and GPx, as a function of malignancy. These results are in agreement with previous evidence showing a similar imbalance in the antioxidant system in melanoma cells as compared with normal melanocytes [20] and in lung cancer as compared with normal lung tissues [21]. We expanded this evidence by correlating the endogenous imbalance in the levels of antioxidant enzymes with the ensuing increase in H_2O_2 and the control of tumor cell proliferation.

However, controversial evidence points towards the involvement of either $O_2^{\cdot-}$ or H_2O_2 in proliferation and transformation. Previous studies reported an association between H_2O_2 and cell proliferation [12–14,42] and the inhibition of proliferation by scavenging H_2O_2 has been reported in a more restricted context, in smooth muscle cells [43,44] and in HER-2/Neu transformed fibroblasts [45]. Regarding the involvement of H_2O_2 in malignant transformation, low catalase levels in tumor cells may play a functional role in the appearance of the malignant phenotype [46–49]. Moreover, high levels of H_2O_2 may give rise to the prooxidant state in tumor cells required for the maintenance of the tumor cell phenotype [48,49]. In this sense, Huang et al. demonstrated the induction of transformation by H_2O_2 via the activation of the epidermal growth factor receptor in rat liver nonneoplastic epithelial cells [9]. Arnold et al. [50] reported that the tranformed phenotype induced by overexpression of Nox1 in NIH 3T3 cell is reverted by stable catalase expression. In this study, a correlation between the endogenous levels of H_2O_2 and malignancy was demonstrated. Moreover, a compensation of an endogenous imbalance of antioxidant enzymes has been shown to inhibit proliferation and revert the malignant phenotype.

Table 3. Characterization of Proliferation and Malignant Features of Stably Transfected MCF-7 Cells With the Full-Length Human Catalase cDNA

Cell lines	Doubling time (h)	Clonogenicity (colonies/10^3 cultured cells)	BrdU incorporation (% positive cells)	Anchorage independence (colonies/10^4 cultured cells)
MCF-7	25 ± 1.8	185 ± 10	44 ± 2.8	65.6 ± 5
AntHCcDNA	23 ± 3.2	190 ± 7	38 ± 1.4	55.6 ± 3.5
SenHCcDNA	53 ± 5*	25 ± 6.3*	19 ± 2.8*	5.6 ± 4.8*

AntHCcDNA, antisense cDNA human catalase; SenHCcDNA, sense cDNA human catalase.
Parental MCF-7 cells and MCF-7 cells transfected with antHCcDNA were used as controls. Stably transfected MCF-7 cells with the full-length human catalase cDNA (senHCcDNA).
*$P < 0.001$ compared with both control conditions.

Regarding the involvement of $O_2^{\cdot-}$ in proliferation, it has been described as mediator of mitogenic signaling in ras-transformed fibroblasts [8]. Moreover, a role for $O_2^{\cdot-}$ in the promotion of neoplastic transformation by TPA in JB6 cells has been described [51,52]. Other authors [15] suggested an inverse correlation between MnSOD levels and malignancy and the MnSOD gene has been hypothesized as a tumor suppressor [53–55]. These studies reported that overexpression of MnSOD suppresses the malignant phenotype [53–55], and that this effect has been reverted by cotransfection of MnSOD with GPx [56]. Moreover, suppression of the malignant phenotype by transfection of CuZnSOD in human glioma cells has been reported [57]. The authors [56,57] suggested that accumulation of H_2O_2 or other hydroperoxides could explain the suppression of tumor growth in MnSOD or CuZnSOD-overexpressing cells by changing the cellular redox status or leading to the production of noxious hydroxyl radicals via the Fenton reaction. In this sense, previous studies [58,59] suggested that H_2O_2 represents a major intracellular ROS in the pathway to cytotoxicity. In addition, Rodriguez et al. [60] reported that overexpression of MnSOD alters mitochondrial function, leading to a decrease in net ATP production and a concomitant decrease in proliferation. An increase in the steady state production of H_2O_2 in these cells overexpressing MnSOD was also demonstrated. Coexpression of catalase reverted the inhibition of proliferation by protecting the cell from the cytotoxicity of H_2O_2 and enhancing net ATP production.

Thus, we suggest that the controversies regarding the role of H_2O_2 in cell proliferation and transformation could be due to concentration-dependent variations [6]. Slight variations in the fine balance between toxicity and the induction of growth-related genes would decide whether the effect of H_2O_2 is growth stimulation or inhibition. Thus, H_2O_2 at different concentrations may induce cell proliferation [42–45], malignant transformation [9,41,46–49], apoptosis [44], senescence, or cytotoxic effects [61].

Further investigations are needed to elucidate whether H_2O_2 could act as an extracellular signaling molecule. In this sense, we demonstrated the inhibition of proliferation not only by decreasing the endogenous intracellular levels of H_2O_2, but also by scavenging extracellular H_2O_2.

In conclusion, we suggest that specific changes in the antioxidant system that metabolize each ROS play a crucial role in the alteration of cell proliferation control, which could be involved in the generation of the malignant phenotype. In particular, high constitutive levels of H_2O_2 in the regulation of cell proliferation sustained by an imbalance in antioxidant enzymes could be a relevant event in the carcinogenesis process that would control the proliferative activity of tumor cells and would be involved in the appearance of malignant features.

Finally, these results suggest the possibility of exploring specific-antioxidant therapies for human tumors employing compounds with H_2O_2-scavenging activity or compensating the antioxidant enzyme imbalance by gene therapy as an alternative to conventional cancer treatment modalities.

REFERENCES

1. Guyton KZ, Kensler TW. Oxidative mechanisms in carcinogenesis. Br Med Bull 1993;49:523–544.
2. Cerutti PA. Prooxidant states and tumor promotion. Science 1985;227:375–381.
3. Kensler TW, Egner PA, Taffe BG, Trush MA. Role of free radicals in tumor promotion and progression. In: Slaga TJ, Klein-Szanto AJP, Boutwell RK, Stevenson DE, Spitzer HL, D'Motto B, editors. Progress in clinical and biological research, Vol. 298. New York: Alan R. Liss; 1989. pp 233–248.
4. Fischer SM, Adams LM. Suppression of tumor promoter-induced chemiluminescence in mouse epidermal cells by several inhibitors of arachidonic acid metabolism. Cancer Res 1985;45:3130–3136.
5. Durán HA, de Rey BM. Differential oxidative stress induced by two different types of skin tumor promoters, benzoyl peroxide and 12-*O*-tetradecanoylphorbol-13-acetate. Carcinogenesis 1991;12:2047–2052.
6. Wolin M, Mohazzab-H KM. Mediation of signal transduction by oxidants. In: Scandalios JG, editor. Oxidative stress and the molecular biology of antioxidant defenses. New York: Cold Spring Harbor Laboratory Press; 1997. pp 21–48.
7. Goldkorn T, Balaban N, Matsukuma K, Chea V, Gould R, Last J, Chan C, Chavez C. EGF-Receptor phosphorylation and signaling are targeted by H_2O_2 redox stress. Am J Respir Cell Mol Biol 1998;19:786–798.
8. Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T, Goldschmidt-Clermont PJ. Mitogenic signaling mediated by oxidants in ras-transformed fibroblasts. Science 1997;275:1649–1652.
9. Huang RP, Peng A, Golard A, Hossain MZ, Huang R, Liu YG, Boynton AL. Hydrogen peroxide promotes transformation of rat liver non-neoplastic epithelial cells through activation of epidermal growth factor receptor. Mol Carcinog 2001;30: 209–217.
10. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J 1996;10:709–720.
11. Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radic Biol Med 1996;21:335–348.
12. Lee SF, Huang YT, Wu WS, Lin JK. Induction of c-jun protooncogene expression by hydrogen peroxide through hydroxyl radical generation and $p60^{SRC}$ tyrosine kinase activation. Free Radic Biol Med 1996;21:437–448.
13. Rao GN. Hydrogen peroxide induces complex formation of SHC-Grb2-SOS with receptor tyrosine kinase and activates Ras and extracellular signal-regulated protein kinases group of mitogen-activated protein kinases. Oncogene 1996;13: 713–719.
14. Burdon RH, Gill V, Alliangana D. Hydrogen peroxide in relation to proliferation and apoptosis in BHK-21 hamster fibroblasts. Free Radic Res 1996;24:81–93.
15. Oberley TD, Oberley LW. Antioxidant enzyme levels in cancer. Histol Histopathol 1997;12:525–535.
16. Solanki V, Rana RS, Slaga TJ. Diminution of mouse epidermal superoxide dismutase and catalase activities by tumor promoters. Carcinogenesis 1981;2:1141–1146.
17. Satomi A, Murakami S, Hashimoto T, Ishida K, Matsuki M, Sonoda M. Significance of superoxide dismutase (SOD) in human colorectal cancer tissue: Correlation with malignant intensity. J Gastroenterol 1995;30:177–182.

18. Lehtola K, Laurikainen L, Leino L, Ahotupa M, Punnonen K. Antioxidant enzymes are elevated in dimethylbenz[a]anthracene-induced neoplastic murine keratinocytes containing an active rasHa oncogene. J Cancer Res Clin Oncol 1995;121: 402–406.
19. Gardner R, Salvador A, Moradas-Ferreira P. Why does SOD overexpression sometimes enhance, sometimes decrease, hydrogen peroxide production? A minimalist explanation. Free Radic Biol Med 2002;32:1351–1357.
20. Picardo M, Grammatico P, Roccella F, Roccella M, Grandinetti M, Del Porto G, Passi S. Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma. J Invest Dermatol 1996;107:322–326.
21. Chung-man Ho J, Zheng S, Comhair SA, Farver C, Erzurum SC. Differential expression of manganese superoxide dismutase and catalase in lung cancer. Cancer Res 2001;61: 8578–8585.
22. Quintanilla M, Haddow S, Jonas D, Jaffe D, Bowden GT, Balmain A. Comparison of *ras* activation during epidermal carcinogenesis in vitro and in vivo. Carcinogenesis 1991;12: 1875–1881.
23. Díaz-Guerra M, Haddow S, Bauluz C, Jorcano JL, Cano A, Balmain A, Quintanilla M. Expression of simple epithelial cytokeratins in mouse epidermal keratinocytes harboring Harvey ras gene alterations. Cancer Res 1992;52:680–687.
24. Larcher F, Robles AI, Durán H, Murillas R, Quintanilla M, Cano A, Conti CJ, Jorcano JL. Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels. Cancer Res 1996; 56:5391–5396.
25. Yuspa SH, Morgan D, Lichti U, Spangler EF, Michael D, Kilkenny A, Hennings H. Cultivation and characterization of cells derived from mouse skin papillomas induced by an initiation-promotion protocol. Carcinogenesis 1986;7:949–958.
26. Gaffney EV. A cell line (HBL-100) established from human breast milk. Cell Tissue Res 1982;227:563–568.
27. Loberg LI, Gauger JR, Buthod JL, Engdahl WR, McCormick DL. Gene expression in human breast epithelial cells exposed to 60 Hz magnetic fields. Carcinogenesis 1999;20:1633–1636.
28. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973;51:1409–1416.
29. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. Science 1977;197:461–463.
30. Beauchamp C, Fridovich I. Superoxide dismutase: Improved assay and an assay applicable to acrylamide gels. Anal Biochem 1971;44:276–287.
31. Nelson DP, Kiesow LA. Enthalpy of decomposition of hydrogen peroxide by catalase at 25 degrees C (with molar extinction coefficients of H_2O_2 solutions in the UV). Anal Biochem 1972;49:474–478.
32. Tappel AL. Glutathione peroxidase and hydroperoxides. Methods Enzymol 1978;52:506–513.
33. Boveris A, Martino E, Stoppani AO. Evaluation of the horseradish peroxidase-scopoletin method for the measurement of hydrogen peroxide formation in biological systems. Anal Biochem 1977;80:145–158.
34. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991;51:794–798.
35. Rook GAW, Steele J, Umar S, Dockrell HM. A simple method for the solubilisation of reduced NBT, and its use as a colorimetric assay for activation of human macrophages by γ-interferon. J Immunol Methods 1985;82:161–167.
36. Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 1997;69: 581–593.
37. Spector DL, Goldman RD, Leinwand LA. Analyzing DNA replication: Nonisotopic labeling. In: Janssen K, editors. Cells. A laboratory manual, Vol. 3. New York: Cold Spring Harbor Laboratory Press; 1998. pp 109.1–109.13.
38. Morgenstern JP, Land H. Advanced mammalian gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 1990;18:3587–3596.
39. McCord JM. Superoxide radical: Controversies, contradictions, and paradoxes. Proc Soc Exp Biol Med 1995;209:112–117.
40. McCord JM. The evolution of free radicals and oxidative stress. Am J Med 2000;108:652–659.
41. Suh YA, Arnold RS, Lassegue B, Shi J, Xu X, Sorescu D, Chung AB, Griendling KK, Lambeth JD. Cell transformation by the superoxide–generating oxidase Mox1. Nature 1999;401: 79–82.
42. Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. Circ Res 1992;70:593–599.
43. Brar SS, Kennedy TP, Whorton AR, Murphy TM, Chitano P, Hoidal JR. Requirement for reactive oxygen species in serum-induced and platelet-derived growth factor-induced growth of airway smooth muscle. J Biol Chem 1999;274:20017–20026.
44. Brown MR, Miller FJ, Jr., Li WG, Ellingson AN, Mozena JD, Chaterjee P, Engelhardt JF, Zwacka RM, Oberley LW, Fang X, Spector AA, Weintraub NL. Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells. Circ Res 1999;85:524–533.
45. Preston TJ, Muller WJ, Singh G. Scavenging of extracellular H_2O_2 by catalase inhibits the proliferation of HER-2/Neu–transformed rat-1 fibroblasts through the induction of a stress response. J Biol Chem 2001;276:9558–9564.
46. Rabilloud T, Asselineau D, Miquel C, Calvayrac R, Darmon M, Vuillaume M. Deficiency in catalase activity correlates with the appearance of tumor phenotype in human keratinocytes. Int J Cancer 1990;45:952–956.
47. Gupta A, Butts B, Kwei KA, Dvorakova K, Stratton SP, Briehl MM, Bowden GT. Attenuation of catalase activity in the malignant phenotype plays a functional role in an in vitro model for tumor progression. Cancer Lett 2001;173:115–125.
48. Sun Y, Colburn NH, Oberley LW. Depression of catalase gene expression after immortalization and transformation of mouse liver cells. Carcinogenesis 1993;14:1505–1510.
49. Ray G, Batra S, Shukla NK, Deo S, Raina V, Ashok S, Husain SA. Lipid peroxidation, free radical production and antioxidant status in breast cancer. Breast Cancer Res Treat 2000;59:163–170.
50. Arnold RS, Shi J, Murad E, Whalen AM, Sun CQ, Polavarapu R, Parthasarathy S, Petros JA, Lambeth JD. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. Proc Natl Acad Sci USA 2001;98:5550–5555.
51. Nakamura Y, Colburn NH, Gindhart TD. Role of reactive oxygen in tumor promotion: Implication of superoxide anion in promotion of neoplastic transformation in JB-6 cells by TPA. Carcinogenesis 1985;6:229–235.
52. Nakamura Y, Gindhart TD, Winterstein D, Tomita I, Seed JL, Colburn NH. Early superoxide dismutase–sensitive event promotes neoplastic transformation in mouse epidermal JB6 cells. Carcinogenesis 1988;9:203–207.
53. Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson PE, Meltzer PS, Trent JM. Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. Proc Natl Acad Sci USA 1993;90:3113–3117.
54. Zhong W, Oberley LW, Oberley TD, St. Clair DK. Suppression of the malignant phenotype of human glioma cells by

overexpression of manganese superoxide dismutase. Oncogene 1997;14:481–490.
55. Lam EW, Zwacka R, Engelhardt JF, Davidson BL, Domann FE, Jr., Yan T, Oberley LW. Adenovirus–mediated manganese superoxide dismutase gene transfer to hamster cheek pouch carcinoma cells. Cancer Res 1997;57:5550–5556.
56. Li S, Yan T, Yang JQ, Oberley TD, Oberley LW. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res 2000;60:3927–3939.
57. Zhang Y, Zhao W, Zhang HJ, Domann FE, Oberley LW. Overexpression of copper zinc superoxide dismutase suppresses human glioma cell growth. Cancer Res 2002;62: 1205–1212.
58. Amstad P, Peskin A, Shah G, Mirault ME, Moret R, Zbinden I, Cerutti P. The balance between Cu, Zn–superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. Biochemistry 1991;30: 9305–9313.
59. Amstad P, Moret R, Cerutti P. Glutathione peroxidase compensates for the hypersensitivity of Cu, Zn–superoxide dismutase overproducers to oxidant stress. J Biol Chem 1994;269:1606–1609.
60. Rodriguez AM, Carrico PM, Mazurkiewicz JE, Melendez JA. Mitochondrial or cytosolic catalase reverses the MnSOD–dependent inhibition of proliferation by enhancing respiratory chain activity, net ATP production, and decreasing the steady state levels of H_2O_2. Free Radic Biol Med 2000; 29:801–813.
61. Davies KJ. Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. IUBMB Life 2000;50:279–289.